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13. ABSTRACT (Maximum 200 Words)

Processing of the N-terminal initiator methionine is an essential cellular process conserved from prokaryotes to eukaryotes. The enzymes that remove N-terminal methionine are known as methionine aminopeptidases. Human methionine aminopeptidase II (hMetAP2) has been identified as the pharmacological target of fumagillin, a natural anti-angiogenic compound originating from fungi. Furthermore, studies on Bengamides, inhibitors of both human MetAPI and II, have revealed significant antitumor effects. These data suggest hMetAP1 may play an important role in tumor cell growth. During the period of this report, a high throughput screen for selective inhibitors of human methionine aminopeptidase I (hMetAP1) was performed. Several selective lead compounds have been identified with a core structure of pyridine-2-carboxylic acid. These compounds also inhibited MCF-7 cell proliferation with micromolar IC_{50} and induced a G2/M phase cell cycle delay. In spite of rescue for cell proliferation by hMetAP1 overexpression, gene-specific silencing (RNAi) of hMetAP1 did not recapitulate the cell cycle effect by drug inhibition. A recent report on mitochondrial hMetAP isoform is in attention and named as hMetAP3. hMetAP3 has been cloned and expressed in *E.coli*. hMetAP1 selective inhibitors showed equal potency toward hMetAP3 in enzymatic assay *in vitro*. Study of hMetAP3 function is currently ongoing.

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Introduction

Processing of the N-terminal initiator methionine is an essential cellular process conserved from prokaryotes to eukaryotes. The enzymes that remove N-terminal methionine are known as methionine aminopeptidases. Human methionine aminopeptidase II (hMetAP2) has been identified as the pharmacological target of fumagillin, a natural anti-angiogenic compound originating from fungi. Furthermore, studies on Bengamides, inhibitors of both human MetAPI and II, have revealed significant antitumor effects. These data suggest hMetAP1 may play an important role in tumor cell growth. During the period of past funding year, a high throughput screen for selective inhibitors of human methionine aminopeptidase I (hMetAP1) was carried out with a 70,000-compound library from ASDI as collaboration. Several non-selective lead compounds have been identified with pyridine core structures. These compounds inhibit cultured cell proliferation in HeLa, MCF-7 and BAEC cells. Meanwhile, rational design has helped to identify a hMetAP1 selective inhibitor with pyridine-2-carboxylic acid core structure. These compounds are 100-fold more selective for hMetAP1 than hMetAP2 in enzymatic assay. They also inhibit HeLa and MCF-7 cell proliferation with micromolar IC_{50}. Further cell cycle analysis has revealed a delay in G2/M phase progression under the inhibitor treatment. Preliminary SAR (structure activity relationship) studies suggested correlation between the cell cycle effect and hMetAP1 enzymatic activity inhibition. Overexpression of hMetAP1 has been shown to partially rescue the anti-proliferative effect in cultured cells without changing the sensitivity to other drugs. Surprisingly, when gene-specific knockdown of hMetAP1 is conducted, there is no significant effect on cell cycle progression. This result puts a second thought about whether hMetAP1 is the physiological relevant target. Interestingly, another group recently reported a mitochondrial specific form of Methionine aminopeptidase (Serero et al., 2003). It is highly possible that this mitochondrial MetAP (named hMetAP3) is also inhibited by hMetAP1-selective inhibitors, and thereafter causing the cell cycle effect. We successfully cloned the mitochondrial MetAP with the N-terminal mitochondria-signal sequence truncated. Recombinant hMetAP3 is purified from *E.coli* with active methionine aminopeptidase activity. hMetAP1-selective inhibitor has been shown to inhibit hMetAP3 with equal potency as hMetAP1 inhibition. Molecular function of hMetAP3 and its effect on cell cycle progression is currently under investigation.

Key accomplishments

- Expressed and purified recombinant GST-tagged human MetAP1 from *E. coli* with kinetic parameters determined.
- Screened a library of 200,000 compounds and identified several non-specific inhibitors for both human MetAP1 and MetAP2 with anti-proliferative activities in different cancer cell lines.
- Rationally designed and synthesized MetAP1-selective inhibitors that block cancer cell proliferation with G2/M phase delay.
- Cloned, expressed and purified recombinant GST-tagged human MetAP3 from *E. coli*.
- Prepared manuscript for X-ray crystallographic study of MetAP1 with Dr. Brian Matthews group at Oregon University.

Statement of Work Task 1: To identify selective and potent inhibitor for human MetAP1 by high throughput screen of chemical library and synthetic chemistry (Months 1-18):

    a. Establishment of high throughput screen system for MetAP1 inhibitors (Months 1-7)

    b. High throughput screen of a diverse 200,000-compound library for potent and selective MetAP1 inhibitory leading compounds (Months1-18)

    c. Studies of structure/activity relationship (SAR) of lead-compound analogs and structural biology of MetAP1 to facilitate systematic chemical modifications of the leading compound (Months 12-24)

Results:

The discovery of bengamides (Fig. 1) as non-selective inhibitors for both human MetAPs has led us to hypothesize the critical function of MetAP1 in tumor cell proliferation (Towbin et al.,

Fig. 1 Chemical structure of Bengamides

2003). To study the functions of MetAP1, we set up a high throughput screening system for identification of MetAP1-selective inhibitors. The
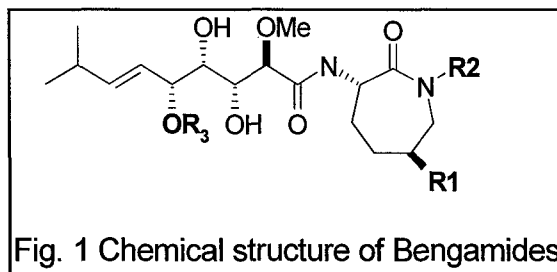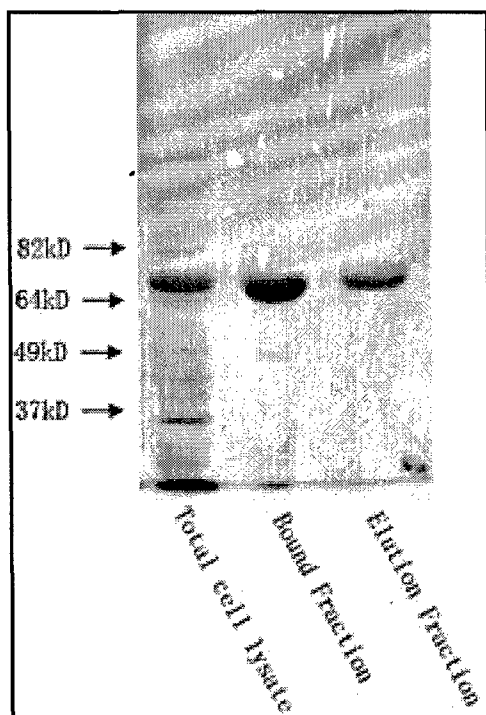
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GST-tagged human MetAP1 recombinant protein was expressed and purified from *E.coli* with relatively high yield (~30mg/L culture) and >90% purity (Fig. 2).

The kinetics study of GST-hMetAP1 is performed with the Proline Aminopeptidase-coupled assay (Zhou et al., 2000). To summarize, the kinetics parameters are: K_m=57.2±8.9 µM, k_{cat} = 0.1325±0.03 s^{-1} and k_{cat}/K_m= 2273 M^{-1}s^{-1}. (Appendix 1).

Fig 2. Expression and purification of GST-tagged MetAP1 from *E.coli*

This purified protein was used for initial screening of a library containing 70,000 compounds in collaboration with ASDI, Inc. A robot from pharmacology department is dedicated for screening these compounds at 10 µM concentration. 8 hits showed IC$_{50}$ < 1 µM and 4 with IC$_{50}$ values in the 1 µM range. Many of these compounds are pyridine-derivatives or triazoles, which have been reported as MetAP inhibitors. When selectivities were tested against MetAP2, almost all of them inhibit MetAP2 equally well with the most difference in IC$_{50}$ value about 12-fold only. These compounds are therefore not able to fit with the criteria of selective inhibitors. Nonetheless, a few pyridine-derivatives have been tested in BAEC (bovine aortic endothelial cells) and HeLa cell cultures with micromolar IC$_{50}$s against cell proliferation.

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In collaboration with Dr. Brian Matthews' group, structural analysis of MetAP1 with pyridine-derivative inhibitor (A602) was successfully obtained (Fig 3). Fascinatingly, A602 compound fits into the active site of MetAP1 facilitated by the Hydrogen-bonds brought in by a third cobalt ion. Although the physiological relevance of this additional metal ion is currently unknown, this crystal structure will help us understand the differences between MetAP1 and MetAP2 active sites and thereafter the drug selectivity. It also indicates a bulky space taken by the third cobalt ion which could be taken advantages for chemical modification of A602 such that additional side chain could be added onto Nitrogen atom(s) on the aromatic ring and increases the association constant between the ligand and receptor.
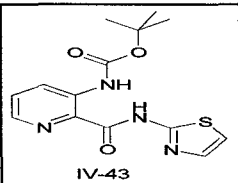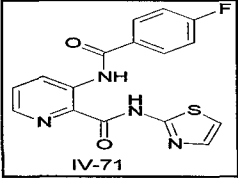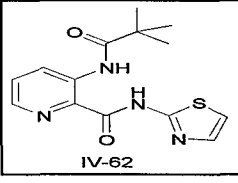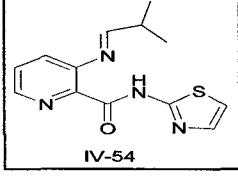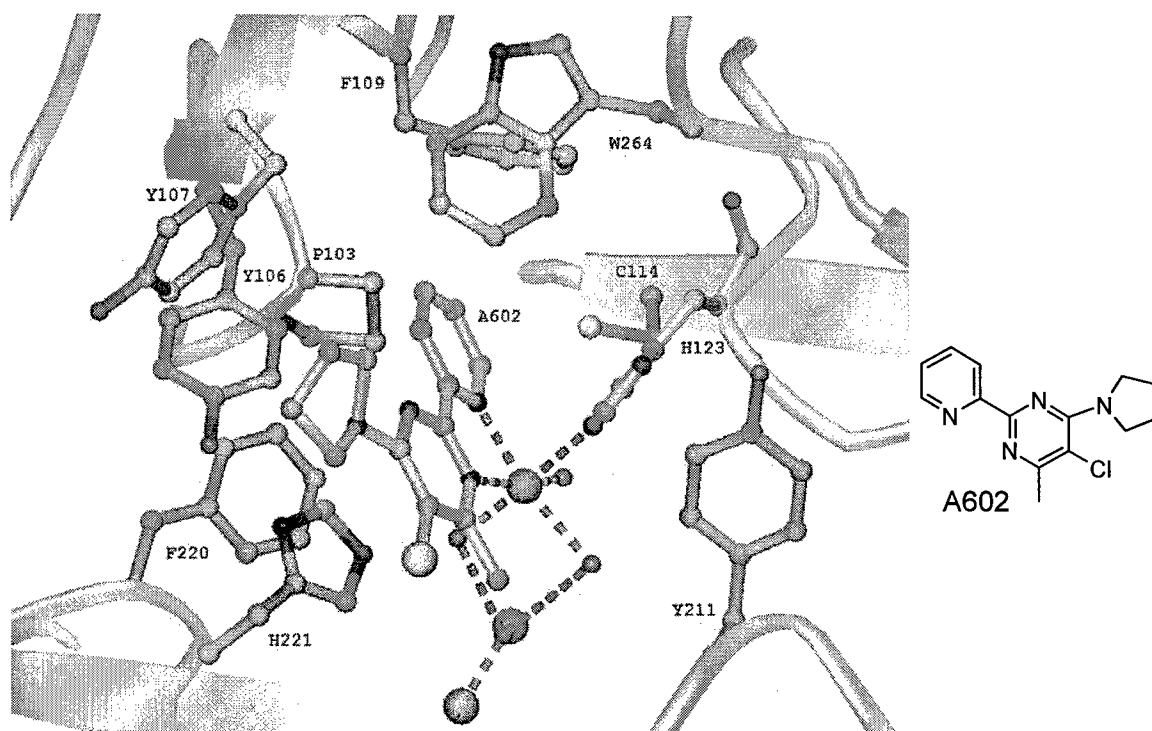

Fig 3. Crystal structure of MetAP1 active site. Chemical structure of A602 compound is shown on the right. A third Cobalt ion (in brown) is present in the pocket to facilitate the H-bonds between A602 and the side chains of H123.

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Since high throughput screen resulted poor MetAP1-selective hits, we resorted to a different approach to attain specific inhibitors for probing its cellular functions. Recently, a new structural class of inhibitors of MetAP1 was reported(Li et al., 2003). This class of compounds shared a common pyridine-2-carbamic acid core structure. We synthesized some structural analogs and tested on both MetAP1 and MetAP2. As shown in Table 1, this class of compounds are highly selective for MetAP1.

Table 1: Representative MetAP1-selective inhibitors. Chemical structures were shown with IC50s for enzymatic assay and MCF-7 and HeLa cell proliferation assays. N/D, not determined.

| | IC_{50} | | | |
	MetAP1	MetAP2	HeLa	MCF-7
IV-43	1.5 μM	>100 μM	2.5 μM	2.3 μM
IV-71	4.9 μM	>500 μM	0.58 μM	N/D
IV-62	2.9 μM	>500 μM	3.9 μM	N/D
IV-54	5.9 μM	> 1 mM	5.8 μM	N/D

To determine the mode of inhibition, we did kinetic studies of MetAP1 in the presence of IV-43 (Appendix 1). The initial velocity was measured and used for data analysis. After Lineweaver-Burk transformations, it shows the increase of drug concentration increased the K_m of MetAP1, but does not

affect the V_{max}. This result suggests that IV-43 is a competitive inhibitor of MetAP1. K_i is subsequently calculated to be 4.1±0.7 μM.

Task 2. To study the physiological functions of human MetAP1 with specific inhibitors. (Months 18-36):

a. Study the functions of MetAP1 in cell cycle progression of breast tumor cell lines by specific MetAP1 inhibition and gene silencing (Months 18-24)
b. Verification of MetAP1 as the physiological target of the inhibitory lead compound (Month 18-30)
c. Investigation of well-documented cellular machinery in cell cycle progression for functional connections with MetAP1 (Months 24-36)
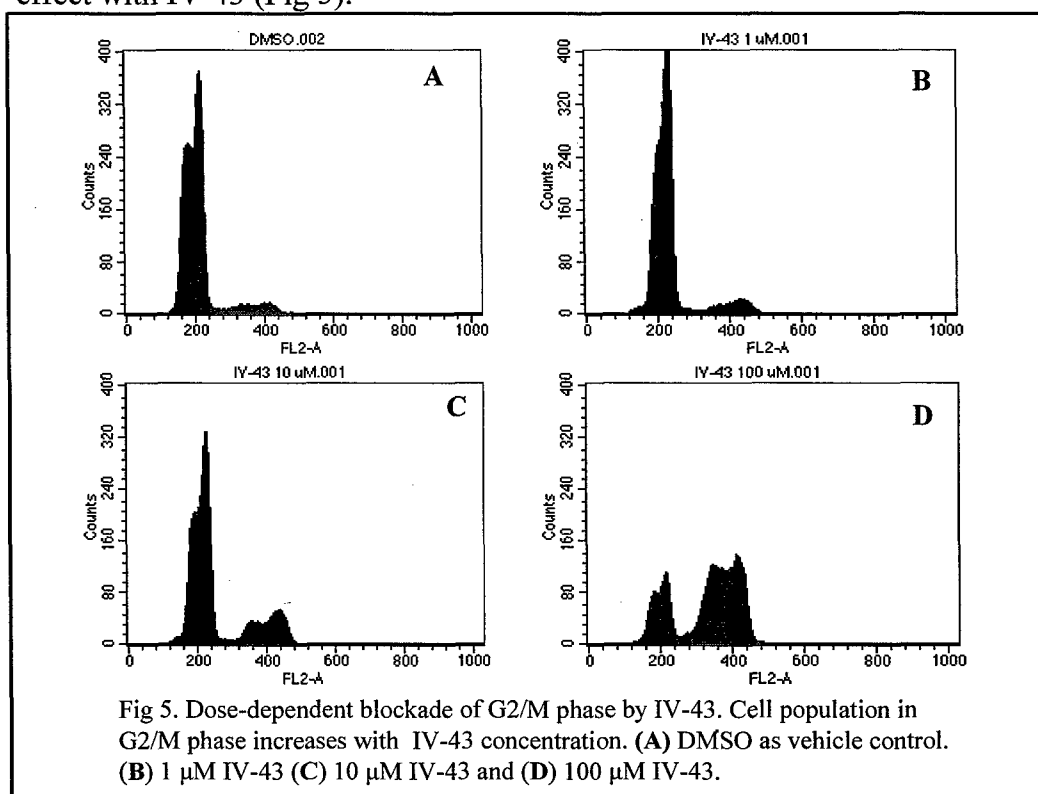
Results:

With the MetAP1-specific inhibitor available, we continue the study of MetAP1 cellular functions. HeLa cells treated with IV-43 compound for 24 hours resulted in blockade of G2/M phase cell cycle progression (Fig 4).
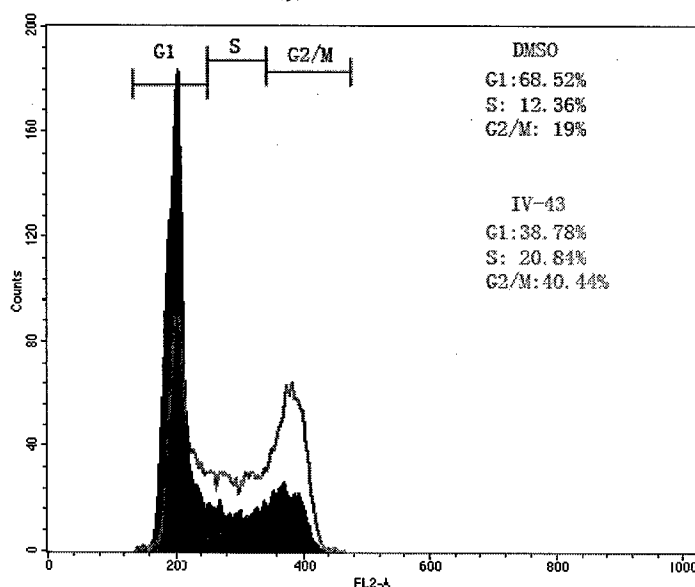
Fig 4. FACS cell cycle analysis for IV-43. 10 μM IV-43 was used to treat HeLa cells for 24 h. The G2/M phase cell population increased significantly than vehicle control.

Further analysis has shown the G2/M phase blockade is a dose-dependent effect with IV-43 (Fig 5).

Fig 5. Dose-dependent blockade of G2/M phase by IV-43. Cell population in G2/M phase increases with IV-43 concentration. **(A)** DMSO as vehicle control. **(B)** 1 μM IV-43 **(C)** 10 μM IV-43 and **(D)** 100 μM IV-43.

To verify MetAP1 is the physiological target of IV-43-related compound, we first overexpressed MetAP1 in HeLa cells and treated both vector-control and MetAP1-overexpression cell populations with IV-71 for cell proliferation assay (IV-71 shares same core structure with IV-43 yet has better anti-proliferative activities in cells)(Wang et al., 2003). Taxol is included as a control for general drug-sensibility (Table 2).

Table 2. Overexpression of MetAP1 increases the IC_{50} of IV-71 treated cells, but not taxol treated cells.

IC50 in HeLa cells

	Vector only	MetAP1 overexpression
Taxol	0.64±0.028 nM	0.66±0.047 nM
IV-71	0.24±0.02 μM	1.2±0.2 μM

It is clear that overexpression of MetAP1 does not change the cell sensibility to other drugs, e.g. taxol. However, it does increase the IC_{50} for IV-71 treatment. A ~6 fold increase of extrogenous MetAP1 protein in overexpression cells has been estimated from Western Blot result (data not shown). This fair correlation between the quantity of MetAP1 overexpression and the increase in IC_{50} indicates that MetAP1 is the physiological target.

Next, we studied IV-43 effect on the N-terminal Methionine level of a specific protein, 14-3-3γ. It has been shown that the N-terminal Methionine of 14-3-3γ protein is a shared substrate for both MetAP1 and MetAP2 (Towbin et al., 2003) Treatment of cells by both fumagillin and bengamide could result the retention of N-terminal Methionine on 14-3-3γ protein. This effect is readily detectable in Western Blot with a monoclonal antibody that recognizes only the N-terminal Methionyl 14-3-3γ protein. HeLa cells were treated with IV-43 for 24h before lysate for Western Blot (Fig 6).
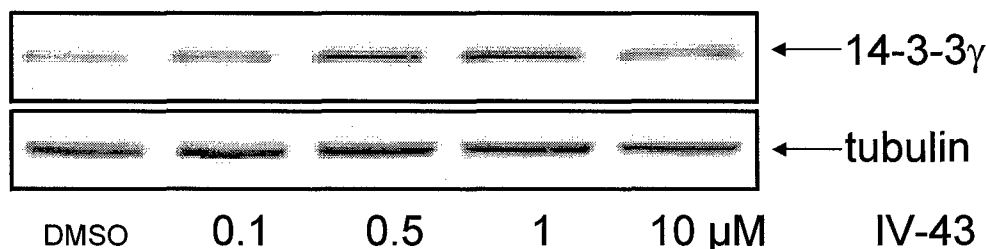

Fig 6. IV-43 increases the N-terminal methionine on 14-3-3γ protein.

As shown above, DMSO vehicle demonstrated the basal level of 14-3-3γ protein with the N-terminal Methionine. Increase in IV-43 concentration revealed a significant increase of the Methionine-14-3-3γ from basal level. This result also suggested that IV-43 inhibit the enzymatic activities of MetAP intracellularly.

To further test the relationship between MetAP1 inhibition and G2/M phase cell cycle blockade, we synthesized an inactive compound (IV-66B) that also shares the core structure with IV-43 with a long side chain. IV-66B shows no activity to either MetAP1 or MetAP2 in enzymatic assay, presumably due to the steric effect caused by the long side chain in enzyme's active pocket. Treatment of cells with IV-66B and another active

compound, IV-71, demonstrated the correlation between MetAP1 inhibition and G2/M phase cell cycle blockade (Fig 7).

IC$_{50}$	MetAP1	> 500µM
	MetAP2	> 500µM

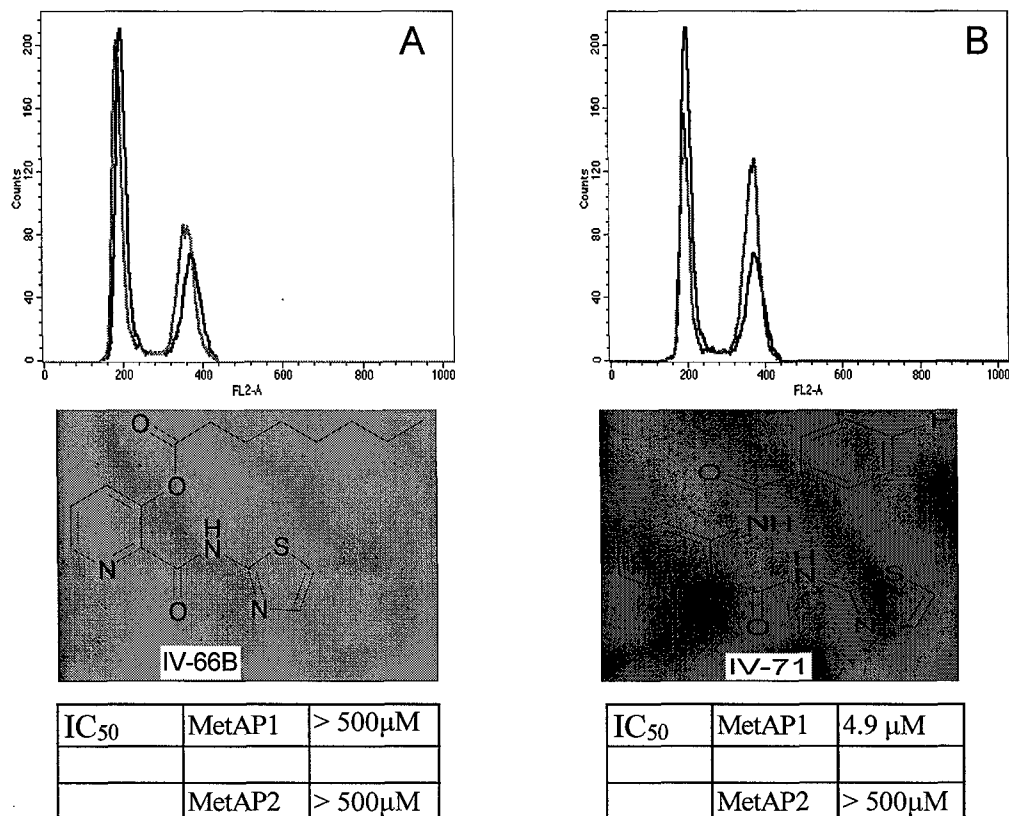| IC$_{50}$	MetAP1	4.9 µM
	MetAP2	> 500µM

Fig 7. Correlation between MetAP1 inhibition and G2/M phase cell cycle blockade. (A) Non-inhibitory compound, IV-66B, treated cells showed no significant difference in cell cycle profile from DMSO vehicle control. (B) MetAP1-specific inhibitor, IV-71, clearly demonstrated a G2/M phase cell cycle blockade from control cell populations. Both control populations are black, IV-66B cell population is in pink and IV-71 cell population in red.

In light of gene-specific silencing technique (RNAi), we made a few constructs to specifically knockdown the gene expression of MetAP1. One construct, namely XH3, is able to knockdown ~90% endogenous MetAP1 in transiently transfected HeLa cells. We further tested MetAP1 RNAi effect on cell cycle progression (Fig 8).
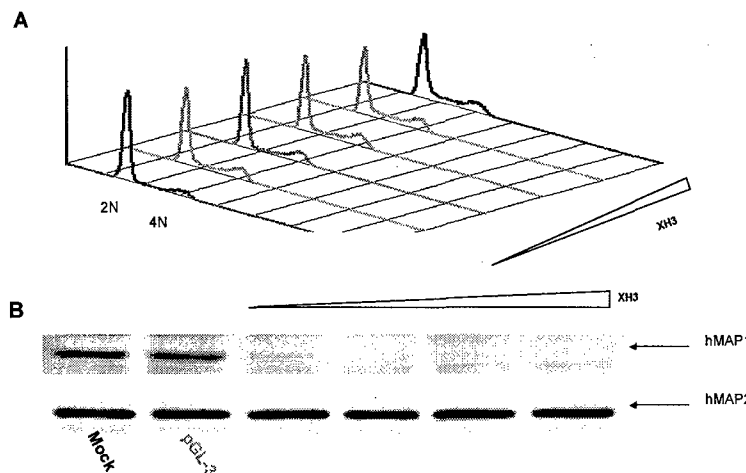
A

B

Fig 8. Transient RNAi of MetAP1 in HeLa cells did not show any significant effect on cell cycle progression. (A) increased dosage of RNAi construct, XH3, did not change the profile of cell cycle progression. (B) Western Blot confirmed the gene-specific silencing of MetAP1 by XH3, dose-dependently. Mock is vector only. pGL-3 stands for scrambled sequence.

To our greatest surprise, MetAP1 RNAi didn't demonstrate any appreciable cell cycle effect from control. This experiment has been done with synchronized HeLa cell populations with similar results (data not shown).

We wondered whether there could be a functionally redundant enzyme in human genome that MetAP1 siRNA did not affect. In fact, it is recently shown that there is a mitochondrial isoform of MetAP (Serero et al., 2003). We tentatively named it human MetAP3. Phylogenic studies have suggested the catalytic domain of MetAP3 is more closely homologous to MetAP1 than MetAP2. In light of this discovery, we hypothesized that IV-43 could inhibit both MetAP1 and MetAP3 in cells. By using RT-PCR technique, we cloned the catalytic domain of MetAP3 (devoid of the putative mitochondria targeting sequence) and made a construct for recombinant expression in bacteria. After induction by IPTG, we successfully expressed and purified the recombinant protein from *E. coli* (Fig 9).
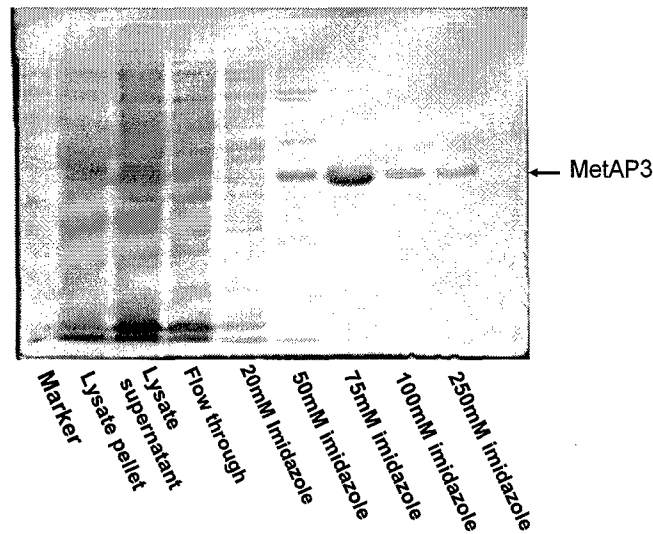
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Fig 9. Expression and purification of recombinant MetAP3
from *E. coli*. The majority MetAP3 was eluted from 75 mM
imidazole fraction.

Next we tested if MetAP3 is indeed a methionine aminopeptidase and
whether IV-43 inhibits MetAP3 activity. As shown in Fig 10, IV-43 inhibits
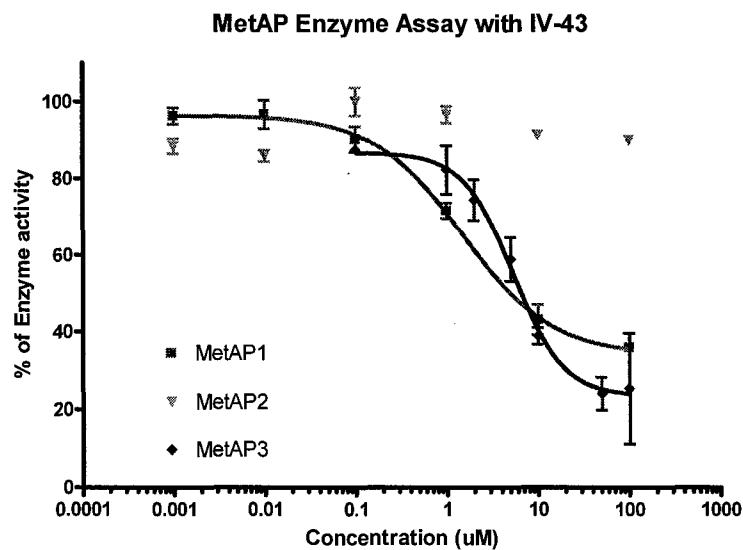both MetAP1 and MetAP3 with micromolar range $IC_{50}s$.

Fig 10. MetAP enzymatic assay with IV-43. Both MetAP1 and MetAP3
were inhibited by IV-43 with micromolar $IC_{50}s$.

We are currently engaging in the study of the cellular functions of MetAP3. Constructs for MetAP3 siRNA are being made and we will use siRNA either alone or in combination of MetAP1 siRNA to assess if both enzymes are responsible for the cell cycle effects caused by IV-43 compound.

Conclusions

The successful expression and purification of MetAP1 and MetAP3 have greatly accelerated the study of their cellular function by means of chemical biology. Both selective and non-selective inhibitors have been identified during this period of research. The synthesis and applications of MetAP1/3-selective inhibitors suggested a physiological function of these enzymes in G2/M cell cycle control. The cloning and preliminary characterization of human MetAP3 has shed light upon further understanding of N-terminal methionine processing in different cellular compartment. The understanding of high-resolution crystal structure of MetAP1 and its complexes with different inhibitors will accelerate the design and synthesis of new classes of MetAP1-specific inhibitors.
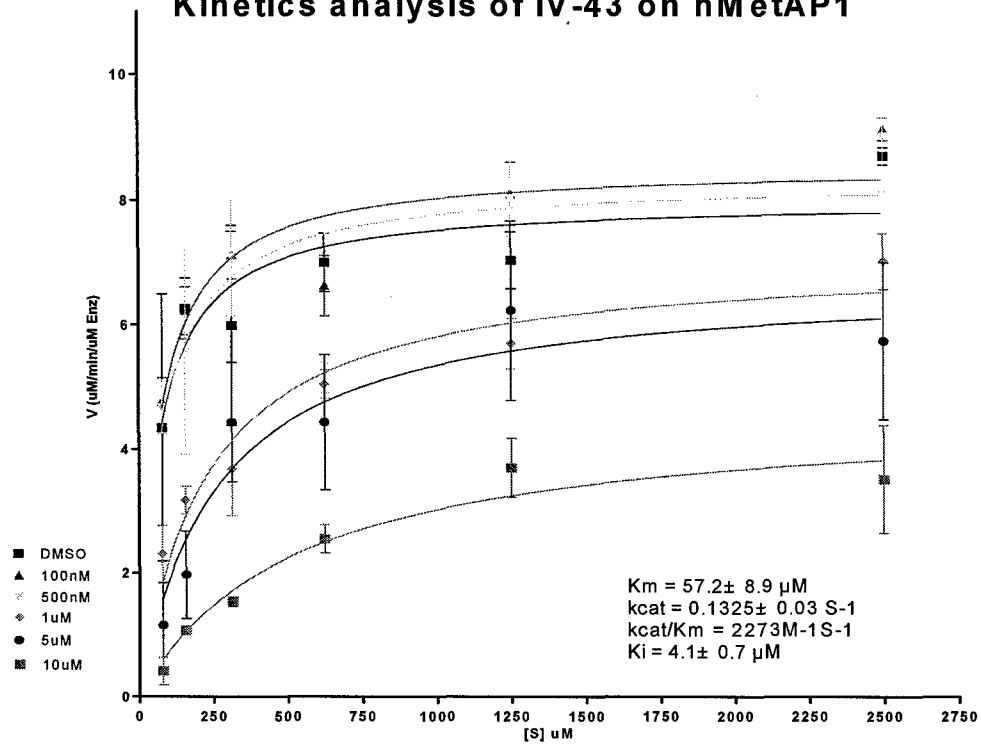
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Appendix 1 Kinetics studies of MetAP1 with IV-43.

Kinetics analysis of IV-43 on hMetAP1

Km = 57.2± 8.9 µM
kcat = 0.1325± 0.03 S-1
kcat/Km = 2273M-1S-1
Ki = 4.1± 0.7 µM

Legend:
■ DMSO
▲ 100nM
✻ 500nM
◆ 1uM
● 5uM
▩ 10uM

Lineweaver-Burk Transformation